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13. ABSTRACT ***(Maximum 200 Words)***

The development of prostate cancer is thought to be driven, at least in part, by the inactivation of as yet unknown tumor suppressor genes, which are manifested as sites of non-random allele loss. Thus, the identification and characterization of these genes is of utmost importance to the understanding of this clinical important malignancy. Our early studies have led to the identification and characterization of *Prk*, a gene coding for a cell cycle serine/threonine kinase. We have mapped the *Prk* gene to chromosome 8p21, a site frequently lost in prostate cancer and proposed to harbor tumor suppressors. We have shown that the *Prk* gene is partially deleted in LACP9AD (prostatic carcinoma), Weri-RB1 (retinoblastoma) and Dami (leukemia) cell lines. *Prk* gene expression, as detected by Northern blotting, is down-regulated in a majority of head and neck cancer. In addition, Prk protein interacts and phosphorylates Cdc25C, a dual specific phosphatase critical for the onset of mitosis. Our studies strongly suggest that a functional loss of Prk, a cell cycle regulator, may contribute to the development of various malignancies including prostate cancer.

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INTRODUCTION

Prostate cancer is the most common malignancy among American men. Yet our understanding of the genetic events which occur during the course of the development of this disease has lagged behind that for other common tumors. Recent studies suggest that prostate cancer appears to be driven by the mutation of as yet unknown genes. The identification of such genes should be essential to the understanding of the biology and pathology of this disease. Allelotype analysis of prostate cancer has implicated several loci of candidate tumor suppressor genes (1-3). For example, loss of heterozygosity of polymorphic markers has been identified at 7q, 8p, 10, 16q and 18q (2,3). Of these, 8p is among the most frequently lost in prostate carcinoma (1-3). Detailed analyses have defined the region of allele loss in prostate and other cancers to 8p12-21 and 8p22-pter (1,4), suggesting that the presence of two or more tumor suppressor genes at this genomic locus.

We have identified a gene, *Prk*, encoding a protein serine/threonine kinase (5-7). Structural analysis has revealed that *Prk* is homologous to the budding yeast cdc5 and *Drosophila melanoganster* polo, both of which have been implicated in regulating G_2/M transition and M phase progression. Mutations of cdc5 in the budding yeast render the yeast unable to complete mitosis at the restrictive temperature. However, this temperature sensitive phenotype can be rescued by the ectopic expression of human *Prk* gene (6). Northern blotting analysis of samples from eighteen lung cancer patients has revealed that *Prk* mRNA is significantly down-regulated in tumor tissues as compared with those of uninvolved tissues (5). FISH analysis has mapped the *Prk* gene to 8p21 (8).

The observations that *Prk* has a function during the cell cycle, similar to known tumor suppressor genes such as RB, p53, and p16, and that it is localized at a site frequently deleted in prostate carcinomas prompted us to test whether *Prk* may be a novel tumor suppressor gene.

RESULTS (Body)

I. Characterization of *Prk* cDNAs derived from prostatic carcinoma as well as other tumor-derived cell lines

IA. Structural alterations. Our preliminary studies have shown that structural abnormalities were detected in several prostate carcinoma cell lines (e.g., DU145, LACP9AD, MCF-7, etc., unpublished studies). To confirm these observations, total RNA was extracted from various tumor-derived cell lines, a first strand cDNA was synthesized and used to amplify the entire *Prk* coding region by polymerase chain reaction (PCR). The PCR products were cloned into pT7Blue vector (Novagen) for sequence analysis. Both strands were sequenced to confirm the sequence alterations. Alterations (deletions) in *prk* sequence were detected in Weri and Dami (Table 1) No mutation was detected in DU145 and MCF-7 (Table 1).

IB. Down-regulation in head/neck squamous cell carcinoma. To examine whether *Prk* expression was altered in head/neck squamous cell carcinomas (HNSCC). Total RNA, extracted from the uninvolved normal tissues and tumor samples of HNSCC patients, was analyzed for *Prk* expression via Northern blotting. We observed that *Prk* mRNA expression is down-regulated in a majority (26 out of 35 patients) of primary HNSCC compared with adjacent uninvolved tissues from the same patients, regardless of stage (Table 2 & Ref. 8). *Prk* transcripts were undetectable in one of the two HNSCC cell lines analyzed (8)

II. Prk interacts and phosphorylates Cdc25C.

To understand the mode of action of Prk protein, it is essential to identify the physiological substrate(s) or regulator(s) of the enzyme. Our extensive studies in the past year have identified one important cell cycle protein (Cdc25C) that apparently is a target of Prk protein in vivo (7, appendix 1). Using several independent but complementary approaches we have demonstrated that Prk physically interacts and phosphorylates Cdc25C. Phospho-peptide mapping analyses indicate that Prk phosphorylates Cdc25C on serine^{216}, a site also phosphorylated by Chk1 and Chk2, two protein kinases involved in DNA damage checkpoint. Thus, our studies point out an intriguing possibility that Prk may be also involved in the DNA damage response pathway.

KEY RESEARCH ACCOMPLISHMENTS

- Deletions were detected in a significant number of tumor-derived cell lines.
- Prk expression was down-regulated in a significant fraction of HNSCC samples.
- Prk interacts with and phosphorylates Cdc25C, a key regulator of cell cycle progression.

REPORTABLE OUTCOMES

1. Ouyang B, Pan H, Li W, Meadows J, Hoffmann I, Dai W. The physical association and phosphorylation of Cdc25C protein phosphatase by Prk. *Oncogene* 1999 28:6029-36.
2. Dai W, Li Y, Ouyang B, Reissmann P, Li J, Wiest J, Stambrook P, Noffsinger A, Bejarano P. *Prk*, a cell cycle protein kinase gene mapped to 8p21, is down-regulated in head and neck cancer. *Genes, Chromosomes, Cancers* 2000 27:332-336

CONCLUSIONS

Inactivation of Prk due to down-regulation of its expression or due to structural abnormalities appears to be linked to several types of malignancies including prostate cancer.

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8. Dai W, Li Y, Ouyang B, Pan H, Reissmann P, Li J, Wiest J, Stambrook P, Cluckman JL, Noffsinger A, Bejarano P. (2000) PRK, a cell cycle gene localized to 8p21, is down-regulated in head and neck cancer. Gene Chromosomes & Cancer 27:332-336.

APPENDICES

A. Reprints

1. Ouyang B, Pan H, Li W, Meadows J, Hoffmann I, Dai W. The physical association and phosphorylation of Cdc25C protein phosphatase by Prk. *Oncogene* 1999 28:6029-6036.
2. Dai W, Li Y, Ouyang B, Pan H, Reissmann P, Li J, Wiest J, Stambrook P, Cluckman JL, Noffsinger A, Bejarano P. (2000) PRK, a cell cycle gene localized to 8p21, is down-regulated in head and neck cancer. Gene Chromosomes & Cancer 27:332-336.

B. Tables 1 & 2

TABLE 1
Prk cDNA Structure in Various Tumor-derived Cell lines

Cell line	Histology	Prk Sequence
DU145	Prostate ca	Mutation not confirmed
LACP9AD	Prostate ca	Deletion
LNCAP	Prostate ca	Under study
MCF-7	Breast ca	Mutation not confirmed
WERI-RB1	Retiboblastoma	Deletion
Dami	Leukemia	Deletion

Table 2. *Prk* Gene Expression in Head and Neck Squamous Cell Carcinoma Patients

	RNA Expression			
Stage	Not Detected	Unchanged	Increased	Decreased
Primary Tumor				
I				2
II	2			7
III	2	1		8
IV	1	2		5
Recurrence			1	4
Total (35)	5 (14%)	3 (8.5%)	1 (2.8%)	26 (74%)

GENES, CHROMOSOMES & CANCER 27:332–336 (2000)

BRIEF COMMUNICATION

PRK, a Cell Cycle Gene Localized to 8p21, Is Downregulated in Head and Neck Cancer

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The human *PRK* gene encodes a protein serine/threonine kinase of the polo family and plays an essential role in regulating meiosis and mitosis. We have previously shown that *PRK* expression is downregulated in a significant fraction of lung carcinomas. Our current studies reveal that *PRK* mRNA expression is downregulated in a majority (26 out of 35 patients) of primary head and neck squamous-cell carcinomas (HNSCC) compared with adjacent uninvolved tissues from the same patients, regardless of stage. In addition, *PRK* transcripts were undetectable in one of the two HNSCC cell lines analyzed. Ectopic expression of *PRK*, but not a *PRK* deletion construct, in transformed A549 fibroblast cells suppresses their proliferation. Furthermore, fluorescence in situ hybridization analyses show that the *PRK* gene localizes to chromosome band 8p21, a region that exhibits a high frequency of loss of heterozygosity in a variety of human cancers, including head and neck cancers, and that is proposed to contain two putative tumor suppressor genes. Considering that *PRK* plays an important role in the regulation of the G2/M transition and cell cycle progression, our current studies suggest that deregulated expression of *PRK* may contribute to tumor development. *Genes Chromosomes Cancer 27:332–336, 2000.*

In the past few years, an emerging family of protein kinases (designated the polo kinase family) has been described in yeast, *Drosophila*, *Xenopus*, mouse, and humans (Sunkel and Glover, 1988; Simmons et al., 1992; Fenton and Glover, 1993; Kitada et al., 1993; Golsteyn et al., 1994; Hamanaka et al., 1994; Donahue et al., 1995; Li et al., 1996; Nigg, 1998). The polo family kinases are important in regulating the onset of mitosis and M-phase progression (Sunkel and Glover, 1988; Fenton and Glover, 1993; Kitada et al., 1993; Nigg, 1998). Mutants of the polo gene in *Drosophila* induce hypercondensed chromosomes and abnormal spindle formation (Fenton and Glover, 1993). A polo homolog encoded by *CDC5* in budding yeast is required for nuclear division in late mitosis (Kitada et al., 1993) and for adaptation to DNA damage response (Toczyski et al., 1997). Recently, it has been shown that a polo-like kinase (*PLX*) from *Xenopus* interacts with, phosphorylates, and activates the *CDC25* gene product (Kumagai and Dunphy, 1996), a dual specific protein phosphatase that, in turn, activates $p34^{cdc2}$. Significantly, PRK protein shares considerable sequence identity with the *CDC5* gene product and is capable of complementing yeast *CDC5* temperature-sensitive mutants (Ouyang et al., 1997), suggesting that it is a functional homolog of the budding yeast *CDC5*.

Neoplastic disease is characterized by a loss of controlled cell proliferation. Deregulated expression of and/or mutations in cell cycle regulatory molecules such as cyclin D1, $p16^{INK4A}$, and RB1 place cells at risk for neoplastic transformation (Sherr, 1996). Cyclin D1, $p16^{INK4A}$, and RB1 participate in regulating the G1 checkpoint and are critical for maintaining the balance between remaining in G1/G0 and commitment to entry into S-phase. Mutations and aberrant expression of genes regulating stages of the cell cycle other than G1 are also found in human cancers. For example, overexpression of cyclin E or cyclin B1 is detected in breast, stomach, endometrium, and colon carcinomas as well as in acute lymphocytic leukemia (Hunter and Pines, 1994; Kamb, 1995). In addition, TP53, a major tumor suppressor known to regulate the G1/S checkpoint, has recently been shown also to be involved in controlling the G2/M checkpoint (Hermeking et al., 1997). Furthermore, the *CHK1* gene product, a serine/threonine kinase, functions as a key component that links the DNA damage check-

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point to mitotic arrest (Peng et al., 1997; Sanchez et al., 1997). The human *CHK1* gene has been mapped to 11q24 (Peng et al., 1997), a locus that has been shown to contain a potential tumor suppressor (Laake and Odegard, 1997).

We have previously shown that *PRK* expression is downregulated in a significant fraction of lung carcinoma samples (Li et al., 1996), suggesting its potential role in regulation of tumor cell growth. To address whether the *PRK* gene is located at a chromosomal locus linked to known human disease, we first obtained from a λ-phage human genomic library a 15-kbp *PRK* gene fragment that was used for mapping the *PRK* gene via the fluorescence in situ hybridization (FISH) method. FISH was performed essentially as previously described (Heng et al., 1992; Heng and Tsui, 1993). Metaphase-arrested lymphocytes were used for analyses. The hybridization efficiency for the *PRK* probe was about 94%, and consistent signals were restricted to chromosome arm 8p and were not detected at other chromosomal regions under the conditions used. An example of the FISH results is shown in Figure 1A and B. The DAPI banding pattern was recorded separately. The assignment of the FISH mapping data with chromosome bands was achieved by superimposing of the *PRK* signals with the same DAPI-banded chromosome, placing the *PRK* gene at 8p21 (Fig. 1C).

Deletions of chromosome arm 8p, as defined by allelic imbalance, are a frequent event in many types of cancers such as those of prostate, head and neck, lung, and colon. This suggests that there exists one (or more) tumor suppressor gene on this chromosome arm. Considering that the steady-state *PRK* mRNA levels are significantly lower in many lung carcinoma samples than in their normal tissue counterparts from the same patients (Li et al., 1996), we asked whether *PRK* expression was also downregulated in some other tumors such as head and neck squamous-cell carcinoma (HNSCC). To this end, 35 HNSCC samples, collected from patients who underwent surgery between October 1992 and March 1995, were used for analyses. Tissues were obtained immediately after surgery and dissected free of necrotic tissues. One part of each specimen was frozen in liquid nitrogen immediately after removal for further RNA study. Normal tissues were taken at least 2 cm away from the tumor, at the resection margin. All specimens and hematoxylin and eosin-stained histologic slides were reviewed by pathologists to identify normal and tumor areas. Histologic examination confirmed that the specimens were comprised of more than 95% tumor cells. Adjacent normal mucosa was also available from each patient. Normal tissues were also evaluated histologically and found to have at

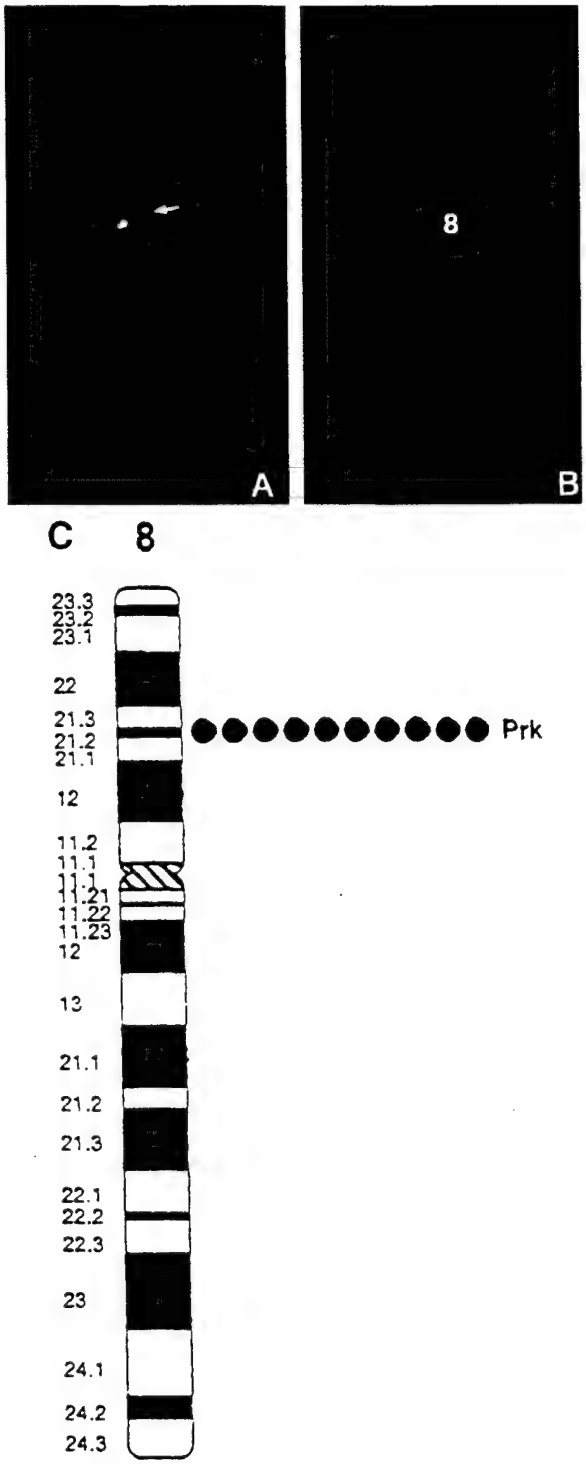

Figure 1. The *PRK* gene localizes to 8p21. **A:** The FISH signals (two yellow dots) on chromosome 8. **B:** The same mitotic figure as shown in A was stained with DAPI, which localizes the *PRK* gene to chromosome 8, subband p21. **C:** Diagram of FISH mapping results for the *PRK* gene. Dots represent the intensity of the signal detected on chromosome 8.

least 95% normal cells. The patients with primary tumors had not received any radiotherapy or chemotherapy. The patients included 26 males and 9 females and had an average age of 56 years (ranging from 31 to 81 years). Of the 35 HNSCC samples used in this study, 30 were primary and 5 were recurrent carcinomas. The primary HNSCCs included 2 stage I, 9 stage II, 11 stage III, and 8 stage IV carcinomas. Tumor sites included the oropharynx (10 patients), oral cavity (10 patients), hypopharynx (2 patients), parapharyngeal space (1 patient), tongue (2 patients), subglottis (1 patient), and supraglottis (9 patients). Histologically, 7 tumors were well differentiated, 24 were moderately differentiated, and 4 were poorly differentiated. Northern blotting analyses revealed that, although the steady-state level of *PRK* mRNA varied greatly among individual patients (Fig. 2A and B), the tumors (odd lanes) consistently expressed less or no detectable *PRK* mRNA levels compared with adjacent uninvolved tissues (even lanes) from the same patients. A summary of *PRK* expression for the 35 HNSCC samples is presented in Table 1. Expression of *PRK* was undetectable in tumor as well as paired uninvolved samples in four patients. Twenty-six tumor samples showed a significant downregulation of the *PRK* mRNA level compared with the uninvolved tissues from the same patients. Downregulation of *PRK* expression does not appear to be associated with tumor stage. Among patients with recurrent tumors, one carcinoma sample showed a significant increase in *PRK* mRNA expression. All specimens were examined for histology, which revealed that although they were comprised predominantly of tumor cells (about 95%), there was some tissue heterogeneity in all samples (data not shown). Many of the tissues samples used in this report were also used for analysis of *TP53* and *RB* expression (Li et al., 1995, 1997).

We further analyzed two squamous-cell carcinoma cell lines derived from head and neck cancers (UN-SCC29 and UM-SCC6), as well as several other tumor-derived cell lines, for *PRK* expression. Cell lines derived from solid tumors included A549 (transformed lung fibroblast, ATCC), Weri-Rb1 (retinoblastoma, ATCC), UM-SCC29 (head and neck carcinoma), UM-SCC6 (head and neck carcinoma), an endothelium tumor-derived cell line (established in-house), a medulla blastoma-derived cell line (established in-house), and HTP-1 (bladder carcinoma, ATCC). UM-SCC6 and UM-SCC27 cell lines were kindly provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). Two additional cell lines of hematopoietic origin

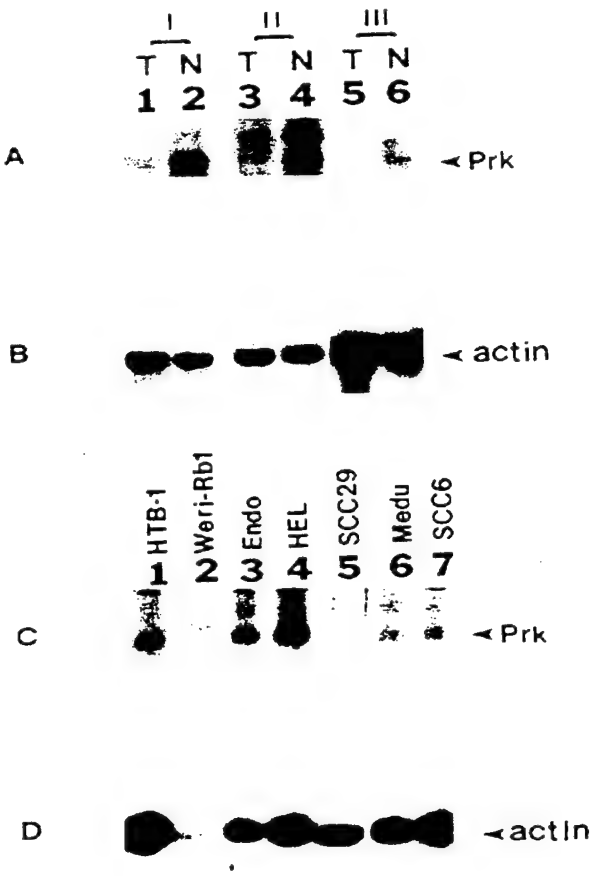

Figure 2. Northern blot analyses of *PRK* mRNA expression. Three examples of paired RNA samples from HNSCC patients were analyzed for *PRK* (**A**) or β-actin (**B**) expression. T stands for tumor-derived RNA and N for uninvolved normal tissues from the same patient. RNAs from various tumor-derived cell lines were analyzed for *PRK* (**C**) and β-actin (**D**) expression. UM-SCC29 and UM-SCC6 are two cell lines established from HNSCC samples.

TABLE 1. *PRK* Gene Expression in Head and Neck Squamous-Cell Carcinoma Patients

	RNA expression			
Stage	Not detected	Unchanged	Increased	Decreased
Primary tumor				
I				2
II	2			7
III	2	1		8
IV	1	2		5
Recurrence			1	4
Total (35)	5 (14%)	3 (8.5%)	1 (2.8%)	26 (74%)

(HEL, erythroleukemia, and Dami, megakaryoblastic leukemia) were also used for analyses. Adherent cell lines were cultured to about 80% confluence before harvesting for RNA analysis. Suspension cell lines were seeded at 2×10^5/ml and collected for RNA analysis when the cells were in logarithmic

growth phase. All cell lines were cultured in media containing 10% fetal calf serum. Northern blotting showed that *PRK* expression varied greatly among the cell lines analyzed (Fig. 2C and D). Little *PRK* expression was detected in UM-SCC29 (Fig. 2C, lane 5), whereas there was a moderate level of *PRK* expression in UM-SCC6. In contrast, the levels of *PRK* expression were very high in cell line HEL (Fig. 2C, lane 4). Interestingly, abnormal *PRK* transcripts (~ 2.9 kb) were detected in cell line Weri-RB1 (Fig. 2C, lane 2).

To determine whether deregulated *PRK* expression affected cell proliferation, we transfected A549 cells with expression constructs that encode either the full length (pcDNA3-PRK) or the short form (kinase inactive, pcDNA3-PRK-St) of *PRK* or with the vector alone (pcDNA3, Clontech, Palo Alto, CA) using lipofectamine (Gibco-BRL, Grand Island, NY) as a transfecting vehicle. The short form of PRK was obtained by removal of 23 N-terminal amino acid residues immediately after the methionine residue (Li et al., 1996). Stable transfectants were pooled, and an equal number of stably transfected cells were cultured in medium containing G418 (250 μg/ml). After 4 days of culture, A549 cells transfected with *PRK* cDNA grew much more slowly compared with those transfected with the vector alone or with the short form of *PRK* (Fig. 3A). Total RNA from each transfected population was also analyzed for *PRK* expression by Northern blotting. Because Dami cells express high levels of *PRK* transcript, DAMI cell RNA was used as a positive control. The near-full-length *PRK* cDNA (Fig. 3B, lane 4) was expressed as expected after transfection into A549 cells. Because the near-full-length *PRK* cDNA contains neither 5′ nontranslated sequence nor 3′ nontranslated sequence encompassing mRNA-destabilizing (ATTTA) motifs, the expressed *PRK* mRNA in transfected cells is shorter (Fig. 3B, lane 4) than its full-length cellular counterpart (lane 1). The proliferation experiment was repeated three times and similar results were obtained. In addition, inhibition of cell growth by constitutive *PRK* expression was also observed with GM00637D cells (data not shown).

The *PRK* gene encodes a protein of the polo kinase family (Li et al., 1996) whose members play a major role in regulating the G2/M checkpoint or mitotic progression (Fenton and Glover, 1993; Kitada et al., 1993; Kumagai and Dunphy, 1996). We have previously shown that the PRK protein level and its kinase activity are tightly regulated during the cell cycle (Ouyang et al., 1997). Many cell cycle regulatory genes (*RB1*, *p16*) or cell cycle

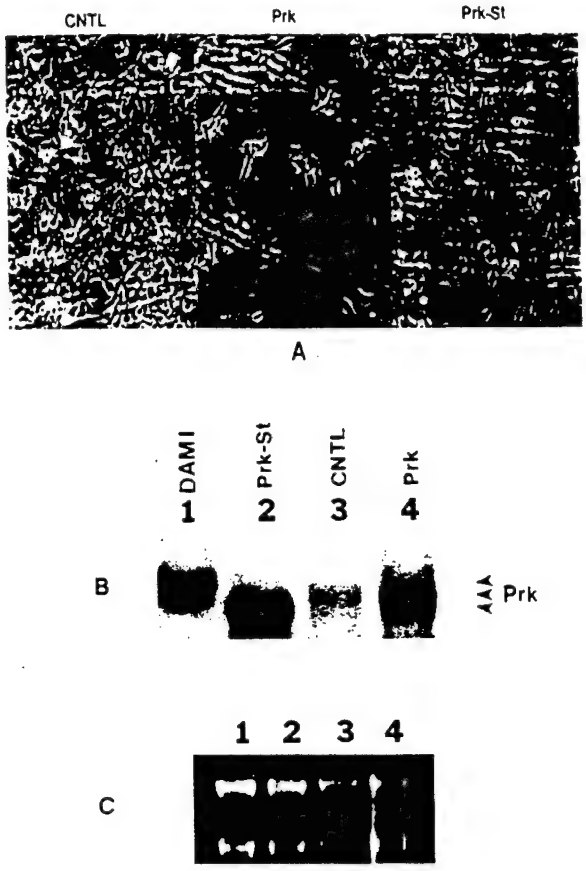

Figure 3. Inhibition of cell proliferation by constitutive expression of *PRK*. **A:** An equal number of A549 cells stably transfected with a full-length *PRK* cDNA (Prk), a truncated *PRK* cDNA (Prk-St), or the vector (CNTL) were seeded in culture plates. After 7 days' culture, overall growth patterns for each were recorded. **B:** Total RNAs from stably transfected A549 cells (lanes 2–4) were analyzed for *PRK* expression. Lane 1 denotes total RNA from megakaryoblastic leukemia Dami cells. **C:** Ethidium bromide staining of the RNA gel shown in B as a loading control.

checkpoint control genes (*TP53*, *ATM*) play a pivotal role in maintaining negative regulation of cell growth. Inactivation of these genes due to mutation, deletion, or their down-regulation often leads to malignant transformation (Sherr, 1996; Xu et al., 1997). We have reported that *PRK* expression is downregulated in a majority of lung carcinoma samples (Li et al., 1997). In the current study, we show that *PRK* is also downregulated in HNSCC, suggesting that compromised expression of *PRK* is correlated with the development of malignancy. Our FISH study reveals that the *PRK* gene localizes to 8p21, a chromosome locus that undergoes LOH in many types of human cancer, including HNSCC and lung carcinomas (Spurr et al., 1995; Bockmuhl et al., 1998), and that appears to harbor two putative tumor suppressor genes (Spurr et al., 1995; Imbert et al., 1996). Considering that constitutive expression of *PRK* significantly suppresses the prolifera-

tion of transformed lung fibroblast cells, it is tempting to speculate that the *PRK* gene may play an important role in regulating the rate of cell proliferation. Several genes, such as *p16* involved in negative regulation of cell proliferation, are significantly downregulated in many types of cancer (Sherr, 1996), and hypermethylation of the *p16* gene promoter is the primary mechanism that renders the gene transcriptionally inactive in tumors (Tasaka et al., 1998). Thus, it would be of considerable interest to examine whether hypermethylation plays a role in downregulating *PRK* expression in HNSCC as well as lung carcinoma patients.

The mechanism by which *PRK*, when overexpressed, exerts its antiproliferation effect remains unclear. It is interesting that *PLK* is capable of malignant transformation of mammalian cells when overexpressed (Smith et al., 1997). Constitutive expression of *PLK* in NIH3T3 cells causes formation of oncogenic foci that grow in soft agar and form tumors in nude mice (Smith et al., 1997). It is possible that *PRK* has a dual function by sensing the completion of genome duplication and initiating mitosis because its kinase activity peaks at late S and G2. If the *PRK* gene function is compromised and the above processes become uncoupled, the cell would undergo premature mitosis with an incompletely duplicated genome. This will lead to genome instability and predispose the cell to oncogenic transformation.

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We thank Dr. Henry Heng for assisting us in mapping the *PRK* gene.

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The physical association and phosphorylation of Cdc25C protein phosphatase by Prk

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prk **encodes a protein serine/threonine kinase involved in regulating M phase functions during the cell cycle. We have expressed His6-Prk and His6-Cdc25C proteins using the baculoviral vector expression system. Purified recombinant His6-Prk, but not a kinase-defective mutant His6-Prk^{K52R}, is capable of strongly phosphorylating His6-Cdc25C *in vitro*. Co-immunoprecipitation and affinity column chromatography experiments demonstrate that GST-Prk and native Cdc25C interact. When co-infected with His6-Prk and His6-Cdc25C recombinant baculoviruses, *sf*-9 cells produce His6-Cdc25C antigen with an additional slower mobility band on denaturing polyacrylamide gels compared with cells infected with His6-Cdc25C baculovirus alone. In addition, His6-Cdc25C immunoprecipitated from *sf*-9 cells co-infected with His6-Prk and His6-Cdc25C baculoviruses, but not with His6-Prk^{K52R} and His6-Cdc25C baculoviruses, contains a greatly enhanced kinase activity that phosphorylates His6-Cdc25C *in vitro*. Moreover, phosphopeptide mapping shows that His6-Prk phosphorylates His6-Cdc25C at two sites *in vitro* and that the major phosphorylation site co-migrates with the one that is phosphorylated *in vivo* in asynchonized cells. Further studies reveal that His6-Prk phosphorylates Cdc25C on serine^{216}, a residue also phosphorylated by Chk1 and Chk2. Together, these observations strongly suggest that Prk's role in mitosis is at least partly mediated through direct regulation of Cdc25C.**

Keywords: Cdc25C; Prk; protein kinase; protein phosphatase; cell cycle

Introduction

Cyclin-dependent kinase (CDK) p34^{cdc2} plays an essential role in cell cycle progression, and its kinase activity is under complicated but very tight regulation during the cell cycle. p34^{cdc2} alone is not active as its protein kinase activity is strictly dependent on association with cyclin B, whose level fluctuates during the cell cycle. p34^{cdc2} also undergoes post-translational modification via reversible phosphorylation. For example, CAK (CDK-activating kinase) phosphorylates p34^{cdc2} on threonine 161, activating the kinase, whereas Wee1 kinase phosphorylates p34^{cdc2} on tyrosine 15 (Tyr15) and threonine 14 (thr14), inactivating the kinase (Feilotter *et al.*, 1992; Norbury *et al.*, 1992). On the other hand, the Cdc25C gene product, a dual specific phosphatase, dephosphorylates p34^{cdc2} on Tyr^{15} and Thr^{14} and, thus, positively regulates p34^{cdc2} kinase activity (Feilotter *et al.*, 1992; Norbury *et al.*, 1992).

In human, three structurally related genes exist in the Cdc25C family (Galaktionov and Beach, 1991); they are named Cdc25C A, B, and C. Cdc25A appears to be involved primarily in G1 progression and G1/S transition (Galaktionov *et al.*, 1996). Cdc25B is essential for cell cycle progression, possibly regulating G2/M transition as a 'starter' phosphatase (Lammer *et al.*, 1998). Among the three Cdc25 family genes, Cdc25C is best characterized. Cdc25C is activated in G2, and its activity is critical for the onset and progression of mitosis (Sadhu *et al.*, 1990). In *Xenopus*, Cdc25C is a phosphoprotein and is heavily phosphorylated by kinases other than Cdc2 and Cdk2 during G2 (Izumi and Maller, 1995).

In the past few years, an emerging family of protein kinases (the polo kinase family) has been described in yeast (Kitada *et al.*, 1993), *Drosophila* (Llamazares *et al.*, 1991), *Xenopus* (Kumagai and Dunphy, 1996), mouse (Simmons *et al.*, 1992; Golsteyn *et al.*, 1995; Donohue *et al.*, 1995), and human (Hamanaka *et al.*, 1994; Li *et al.*, 1996; also see recent review Nigg, 1998). The *Drosophila* polo gene, homologous to the budding yeast Cdc5, encodes a serine/threonine (Ser/Thr) kinase and is required for mitosis in this species; mutations in this gene result in abnormal mitotic and meiotic division (Fenton and Glover, 1993). The abnormalities of these mutants are manifested as over-condensed chromosomes, abnormal spindle formation, and polyploidy (Llamazares *et al.*, 1991; Fenton and Glover, 1993). *polo* transcripts are abundantly expressed in tissues and developmental stages that display extensive mitotic activity. The polo kinase activity peaks cyclically at anaphase/telophase (Llamazares *et al.*, 1991; Fenton and Glover, 1993).

The human has at least three genes, *plk* (Hamanaka *et al.*, 1994), *prk* (Li *et al.*, 1996) and *snk* (GenBank accession #AF059617), whose structures are related to *Drosophila*. We have previously reported the cloning and characterization of a cDNA coding for human *prk* (proliferation-related kinase or polo-related kinase) (Li *et al.*, 1996). Subsequently, we demonstrate that Prk is involved in regulating M phase functions (Ouyang *et al.*, 1997). Our current studies report Prk's mode of action during cell cycle regulation. We show that Prk specifically interacts with and phosphorylates Cdc25C protein phosphatase.

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Results

Our early studies have demonstrated the role of *prk* in the regulation of mitosis and meiosis (Ouyang *et al.*, 1997). Our preliminary study also shows that Prk phosphorylates Cdc25C *in vitro* (Ouyang *et al.*, 1997). To study the mode of action of Prk during cell cycle regulation, we expressed both human His6-Prk and His6-Cdc25C in insect *sf*-9 cells through the baculoviral vector expression system. Figure 1a shows that Prk antigen is expressed in *sf*-9 cells infected with either His6-Prk baculovirus (lane 5) or His6-Prk/His6-Cdc25C baculoviruses (double-infection, lane 3). The same blot was stripped and reprobed with the anti-Cdc25C antibody. Figure 1b shows that Cdc25C antigen is detected in His6-Cdc25C baculovirus-infected cells (lane 4) or the double-infected cells (lane 3). We also expressed His6-tagged Cdc25C in *E. coli* using a prokaryotic expression vector. As shown in Figure 1b, Cdc25C was highly induced after IPTG addition (lane 1) as compared with the non-induced bacterial lysates (lane 2). Figure 1c illustrates the Commassie blue stained blot as a loading control.

We purified recombinant His6-Prk and His6-Cdc25c proteins using affinity chromatography. Purified His6-Prk was analysed for *in vitro* kinase activity using casein or His6-Cdc25C as a substrate. Figure 2a shows that His6-Prk strongly phosphorylated casein (CSN, lanes 1 and 4) but not histone H1 (data not shown) *in vitro*. His6-Prk also phosphorylated the purified His6-Cdc25C *in vitro* (Figure 2a, lanes 3 and 4). To determine whether His6-Prk phosphorylates other members of the Cdc25 family, we expressed and purified GST-Cdc25A and GST-Cdc25B. An equal amount of the purified Cdc25 proteins were used for *in vitro* kinase assays. It was shown (Figure 2b) that His6-Prk phosphorylated all three GST-Cdc25 proteins *in vitro*. It appears that His-Prk phosphorylated Cdc25C (lane 3) more efficiently than it did Cdc25A (lane 1) or Cdc25B (lane 2). To eliminate the possibility that a contaminated protein kinase activity was co-purified with His6-Prk and, was thus actually responsible for the phosphorylation of Cdc25 proteins, we changed a conserved lysine residue (amino acid #52, Ouyang *et al.*, 1997) in the kinase domain of His6-Prk into an arginine residue via site-directed mutagenesis. The mutated molecule His6-Prk^{K52R} was expressed, purified, and assayed for its kinase activity in the same manner as was the wild-type His6-Prk. Figure 2c shows that His6-Prk^{K52R} weakly phosphorylated His6-Cdc25C (lane 4) whereas His6-Prk phosphorylated His6-Cdc25C efficiently (lane 3), indicating that Prk is the primary kinase that phosphorylates the phosphatase. Titration experiments confirmed that Prk^{K52R} had a

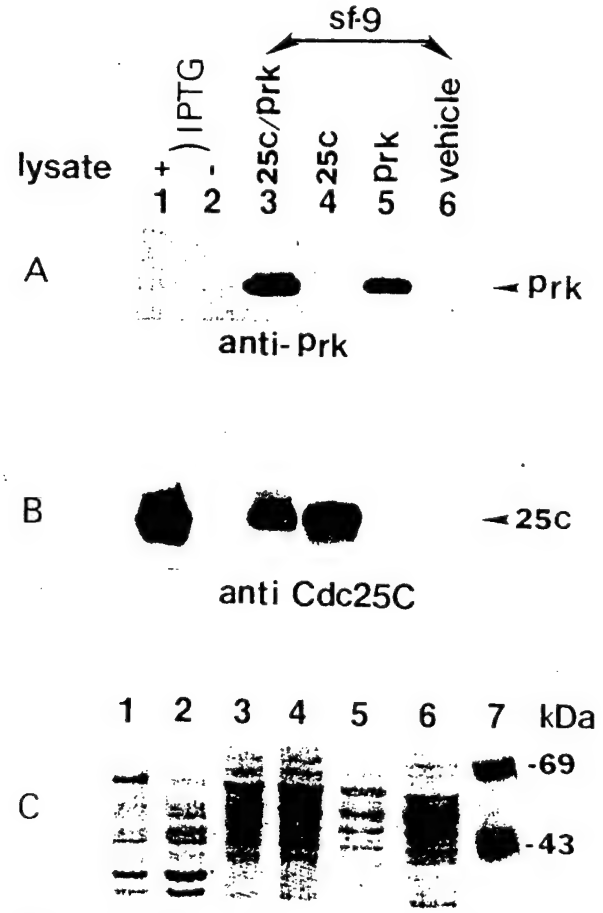

Figure 1 Analysis of human His6-Prk and His6-Cdc25C expression in *sf*-9 cells. *sf*-9 cells infected with the wild-type baculovirus (vehicle), His6-Prk, His6-Cdc25C, or His6-Prk/His6-Cdc25C recombinant baculoviruses were analysed for Prk (a) or Cdc25C (b) expression through Western blotting. His6-Cdc25C was also expressed using a prokaryotic expression vector in the presence (lane 1) or absence (lane 2) of IPTG. (c) Commassie blue staining of the protein blot as a loading control

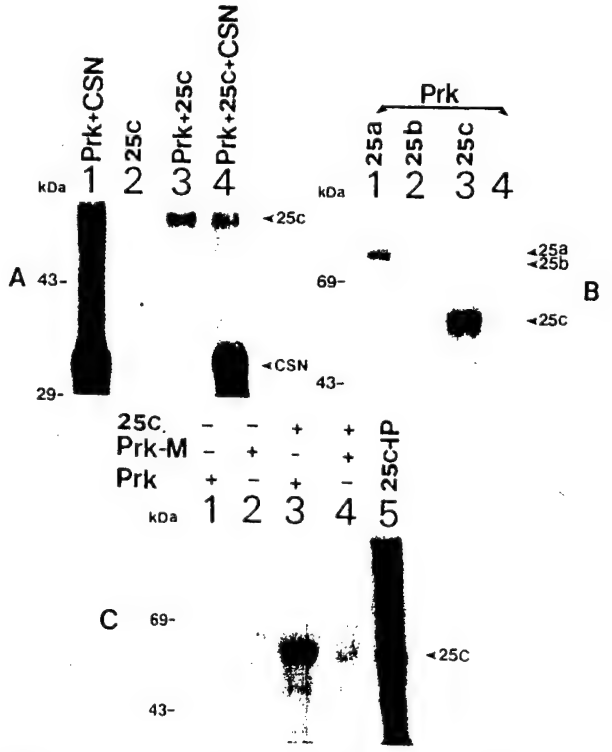

Figure 2 Phosphorylation of His6-Cdc25C by His6-Prk. (a) Purified His6-Prk was assayed for its *in vitro* kinase activity towards Cdc25C (2.5 μg/reaction) and casein (15 μg reaction) in the presence of [γ-^{32}P]ATP. The reaction mixtures were fractionated on a denaturing polyacrylamide gel followed by autoradiography. (b) An equal amount (5 μg reaction) of Cdc25A, GST-Cdc25B, and Cdc25C were phosphorylated *in vitro* by His6-Prk. (c) Phosphorylation of Cdc25C by His6-Prk or His6-Prk^{K52R} (Prk-M). Lane 5 (25C-IP) represents Cdc25C immunoprecipitated from ^{32}P-labeled PC-3 cell lysates

residual kinase activity (about 1/5 of His6-Prk, data not shown). It appears that a small fraction of His6-Cdc25C was hyper-phosphorylated and migrated slightly more slowly (Figure 2c, lane 3) as compared with the Cdc25C immunoprecipitated from metabolically labeled Cdc25C (Figure 2c, lane 5).

Many physiological substrates of protein kinases form a relatively stable complex with the enzymes, which can be detected by co-immunoprecipitation. To determine whether Prk and Cdc25C proteins directly interact with each other, we immunoprecipitated protein lysates from *sf*-9 cells infected with His6-Prk or His6-Cdc25C or both recombinant baculoviruses. Immuno-precipitates were analysed by SDS–PAGE followed by Western blotting using the anti-Prk antibody. Figure 3a and b show that His6-Cdc25C immunoprecipitated from double-infected cells (lane 4), but not from cells infected with the wild-type, His6-Prk or His6-Cdc25C baculovirus (lanes 1–3), contained Prk antigen. Further experiments were performed in which various cell lysates were immunoprecipitated with the anti-Prk antibody or control antibodies (anti-Cdc25C as a positive control or anti-β-actin as a negative control). Immunoprecipitates were then analysed for the presence of Cdc25C antigen by Western blotting. It was observed (Figure 3c) that the anti-Prk antibody (lane 1) but not the control antibody (lane 5) brought down Cdc25C antigen. The same blot was stripped and reprobed with the anti-Prk antibody, and it was shown that immunoprecipitates of Cdc25C (Figure 3d, lane 3) but not β-actin (lane 5) contained the Prk antigen.

To ascertain whether Prk interacts with native Cdc25C, we expressed the C-terminal half of Prk as a GST-fusion protein. GST-Prk, as well as control

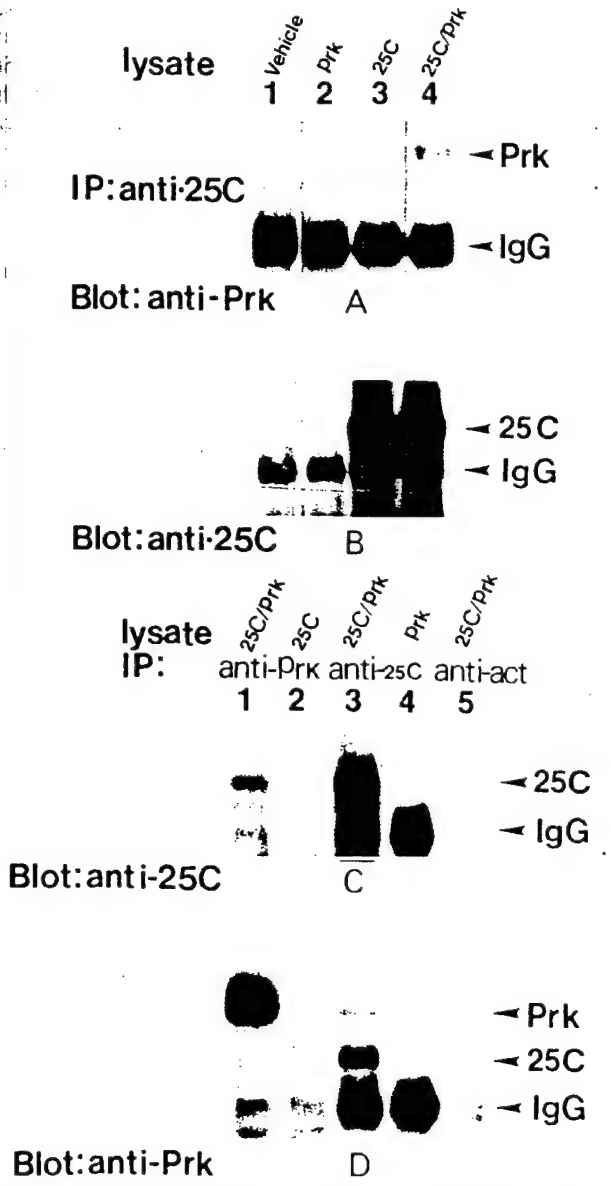

Figure 3 Physical association between His6-Prk and His6-Cdc25C. (**a**) Various *sf*-9 cells infected with the wild-type baculovirus (vehicle), His6-Prk, His6-Cdc25C, or His6-Prk His6-Cdc25C recombinant baculoviruses were immunoprecipitated with the anti-Cdc25C antibody. Cdc25C immunoprecipitates were analysed by Western blotting using the anti-Prk antibody. (**b**) The blot as shown in a was stripped and reprobed with the anti-Cdc25C antibody. (**c**) Various *sf*-9 cell lysates were immunoprecipitated with the anti-Prk (lanes 1 and 2), the anti-Cdc25C (lanes 3 and 4), or the anti-β-actin (lane 5) antibody. Immunoprecipitates were analysed for Cdc25C expression through Western blotting. (**d**) The same blot as shown in c was stripped and reprobed with the anti-Prk antibody

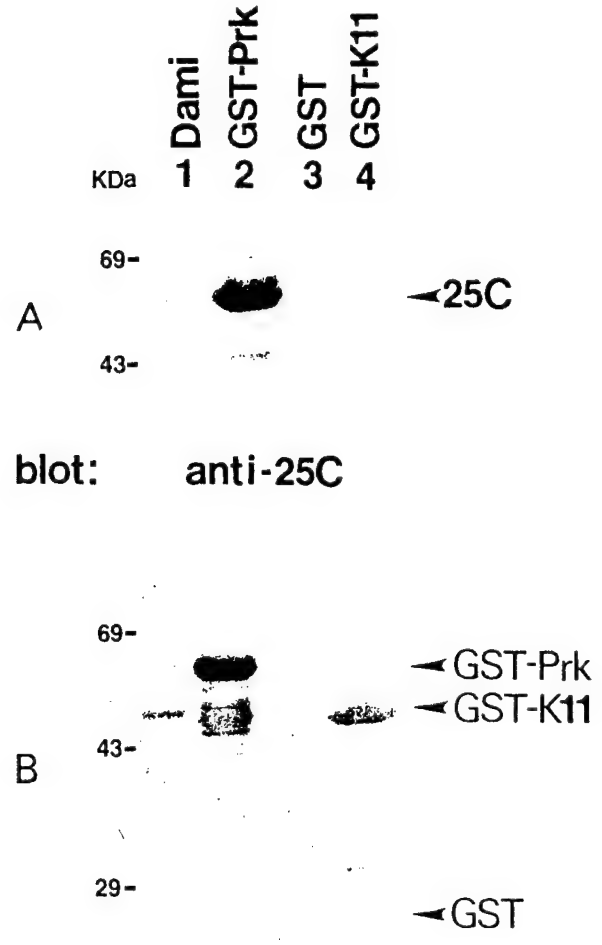

Figure 4 Physical association of GST-Prk with native Cdc25C molecule from Dami Cells. (a) GST-Prk proteins as well as control proteins GST or GST-K11 were immobilized on to glutathione resins which were subsequently incubated with protein lysates from Dami cells. After thorough washes, proteins eluted from GST-Prk, GST alone, or GST-K11 were analysed for the presence of the Cdc25C antigen through Western blotting. (**b**) The same blot as shown in a was stained with Coomassie brilliant blue

proteins GST-K11 (an RNA-binding factor) or GST, were immobilized to glutathione Sepharose resins as described in the Experimental procedures. Cell lysates (1×10^8 cell equivalent) of Dami cells were incubated with the immobilized GST-Prk, GST-K11, or GST protein (50 μg each). After incubation, the Sepharose resins were thoroughly washed. Proteins bound to GST-Prk, GST-K11 or GST resins were eluted and analysed by SDS–PAGE followed by Western blotting using the anti-Cdc25C antibody. Figure 4a shows that Cdc25C antigen was detected in GST-Prk eluent (lane 2), but not in the eluent of GST alone (lane 3) or GST-K11 (lane 4), indicating that Cdc25C specifically interacts with the C-terminal half of Prk. To confirm that approximately equal amounts of each 'bait' protein were used in the binding, the same blot was stained with Commassie brilliant blue (Figure 4b). It was noted that roughly equal amounts of GST-Prk, GST, or GST-K11 protein were used in the binding although GST-Prk and GST-K11 (Figure 4b, lanes 2 and 4) were partially degraded.

To further determine whether endogenous Prk and Cdc25C interact with each other at a physiological concentration, Cdc25C immunoprecipitates from PC-3 cells were analysed for the presence of Prk antigen by Western blotting. Figure 5a shows that anti-Cdc25C antibody (lane 2) but not a control antibody (lane 1) brought down the Prk antigen. We were unable to detect Cdc25C antigen in Prk immunoprecipitates via Western blotting. We suspected that the anti-Prk antibody may somehow interfere with Cdc25C's association with Prk, and thus reduce interaction efficiency of Cdc25C protein with Prk during immunoprecipitation. To increase detection sensitivity, we metabolically labeled PC-3 cells with ^{32}P-phosphate. Immunoprecipitation of labeled lysates shows (Figure 5b) that the anti-Prk antibody, but not the anti-β-actin antibody (data not shown), is capable of bringing down a protein co-migrating with *in vitro* phosphorylated His6-Cdc25C (lane 1) or Cdc25C immunoprecipitated from PC-3 cell lysates (lane 3). This suggests that Cdc25C physically interact with Prk.

The question of whether Prk phosphorylates Cdc25C was further studied by immunoprecipitating His6-Cdc25C from various *sf*-9 cell lysates using an anti-Cdc25C antibody. The immunoprecipitates were analysed by Western blotting for Cdc25C expression. It was observed that there was a new Cdc25C antigen, with a slow mobility, in doubly-infected cell lysates (Figure 6a, lanes 4 and 6). This Cdc25C antigen was not present in *sf*-9 cells infected with Cdc25C baculovirus alone (Figure 6a, lane 3), suggesting the slow-mobility Cdc25C antigen is a phosphorylation product of Prk. A pretreatment of cell lysates with protein phosphatase converted the slow-moving bands into the fast-moving one (data not shown). To confirm that Cdc25C is associated with Prk *in vivo* and that Cdc25C-associated kinase is capable of phosphorylating the protein phosphatase, His6-Cdc25C immunoprecipitated from equal amounts of cell lysates co-infected with His6-Prk or with His6-Prk^{K52R} baculoviruses was directly incubated with [γ^{32}P]ATP in the kinase buffer. After incubation, the reaction mixture was analysed by SDS–PAGE followed by autoradiography. Figure 6b shows that His6-Cdc25C was phosphorylated strongly in cells co-infected with His6-Prk baculoviruses (lane 1) but weakly in cells co-infected with His6-Prk^{K52R} baculoviruses (lane 3).

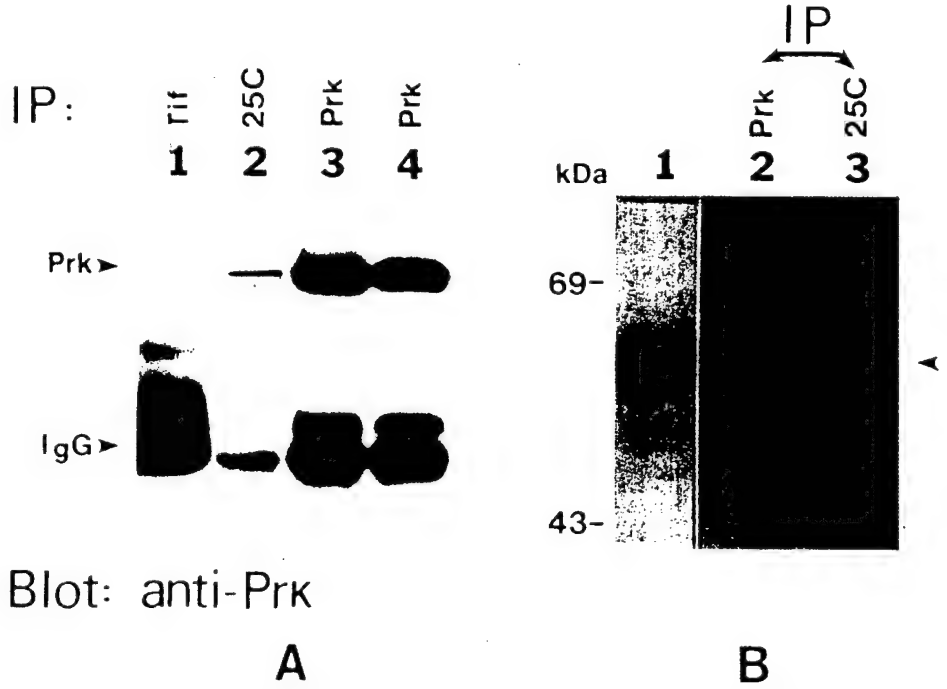

Figure 5 Prk interacts with endogenous Cdc25C. (**a**) PC-3 cell lysates were used for immunoprecipitation with an anti-*tif* (a polyclonal antibody against receptor tyrosine kinase *tif* (lane 1), anti-Cdc25C (lane 2), and anti-Prk (lanes 3 and 4, replicates) antibodies. Immunoprecipitates were blotted for the presence of Prk antigen. (**b**) ^{32}P-labeled PC-3 cell lysates were immunoprecipitated with an anti-Prk (lane 2) or anti-Cdc25C (lane 3) antibody. Immunoprecipitates were fractionated on a SDS polyacrylamide gel followed by autoradiography. Lane 1 represents His6-Cdc25C protein phosphorylated *in vitro* in the presence of [γ^{32}P]ATP by His6-Prk as a standard

Western blotting showed that both His6-Prk and His6-Prk^{K52R} were expressed at similar levels in these cells (data not shown). This suggests that Prk was the primary kinase phosphorylating His6-Cdc25C. Additional experiments were also performed in which various *sf*-9 cell lysates were immunoprecipitated with the anti-Prk antibody, and Prk immunoprecipitates were analysed for kinase activity without addition of exogenous substrates. It was observed that His6-Prk, immunoprecipitated from doubly-infected *sf*-9 cells or the cells infected with His6-Prk baculovirus alone, was autophosphorylated (Figure 6c, lanes 3 and 4). As expected, a protein migrating at about 60 kDa was co-immunoprecipitated with, and phosphorylated by His6-Prk in doubly-transfected (25C/Prk, lane 4). No such protein was present in cells infected with the wild-type (lane 1), His6-Prk (lane 3), or His6-Cdc25C (lane 2) baculovirus alone, strongly suggesting that Cdc25C is an *in vivo* substrate of Prk.

To determine whether Prk phosphorylates Cdc25C at physiologically relevant sites, Cdc25C purified from metabolically-labeled asynchronized PC3 cells, as well as His6-Cdc25C phosphorylated *in vitro* by His6-Prk, were subjected to peptide mapping as described in the Experimental procedures. Figure 7a shows that the phosphopeptide map of His6-Cdc25C phosphorylated by His6-Prk *in vitro* yielded two discrete spots. This map was similar to the one obtained with Cdc25C phosphorylated *in vivo* (Figure 7a). Mixing experiments showed that the major spot from *in vitro* experiments superimposed with the spot of *in vivo* experiments, strongly suggesting that His6-Prk phosphorylated His6-Cdc25C at a site that is also phosphorylated *in vivo*. It is known that the major phosphorylation site on Cdc25C in asynchronized cells is serine^{216} (Ogg *et al*., 1994). Therefore, we used an equal amount of purified mutant GST-Cdc25C^{S216A}(200–256) peptide motif with serine^{216} converted to alanine as well as the wild-type GST-Cdc25C(200–256) peptide motif as *in vitro* substrates of His6-Prk. These peptides were originally used for assays for Chk1 and Chk2 protein kinase activities as reported (Sanchez *et al*., 1997; Matsuoka *et al*., 1998). Figure 7b shows that His6-Prk phosphorylated very poorly, if any, GST-Cdc25C^{S216A}(200–256) *in vitro* (lanes 2 and 3) whereas it strongly phosphorylated GST-Cdc25C(200–256) (lane 1). Moreover, His6-Prk^{K52R} only weakly phosphorylated the wild-type GST-Cdc25C(200–256) motif (Figure 7, lane 4), indicating that Prk phosphorylated Cdc25C on serine^{216}.

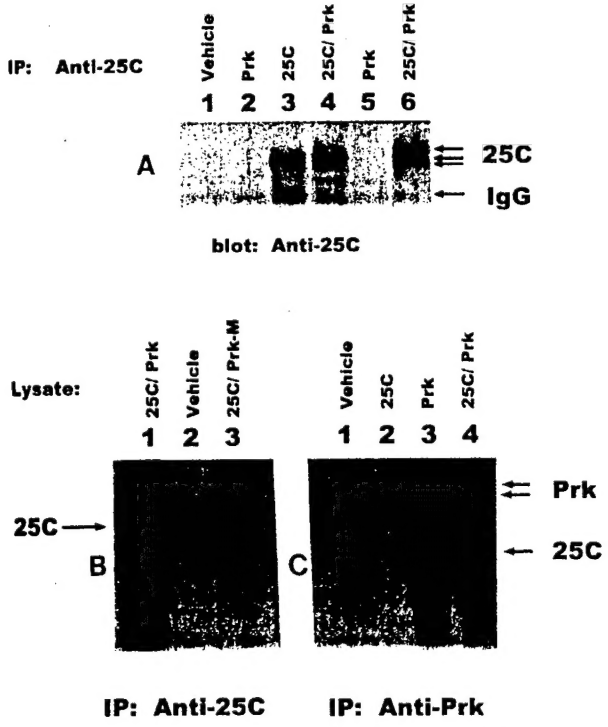

Figure 6 Phosphorylation of His6-Cdc25C by His6-Prk *in vivo*. (**a**) Various *sf*-9 cell lysates were immunoprecipitated with the polyclonal anti-Cdc25C antibody, and the immunoprecipitates were analysed by Western blotting using the monoclonal anti-Cdc25C antibody. Lanes 5 and 6 are duplicates of lanes 2 and 4 from independent experiments. (**b**) *sf*-9 cells doubly-infected with His6-Cdc25C and His6-Prk baculoviruses (lane 1), or with His6-Cdc25C and His6-Prk^{K52R} baculoviruses (lane 3), were lysed and equal amounts of cell lysates were immunoprecipitated with the anti-Cdc25C antibody. Immunoprecipitates were directly analysed for protein kinase activities in the presence of [γ^{32}P]ATP without addition of exogenous substrates. The kinase reaction mixtures were fractionated on denaturing polyacrylamide gel followed by autoradiography. (**c**) Various *sf*-9 cell lysates were immunoprecipitated with the anti-Prk antibody (lanes 1–4). The immunoprecipitates were subjected to *in vitro* kinase assays without addition of exogenous substrates followed by SDS–PAGE and autoradiography as described in B

Discussion

Several previously characterized protein kinases have been implicated in phosphorylating and regulating the activity of Cdc25C. It has been shown that Plx1, a *Xenopus* Plk, physically associates with and phosphor-

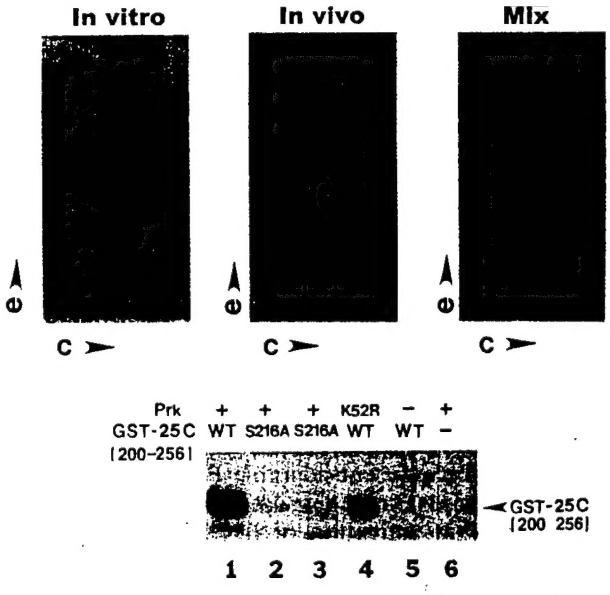

Figure 7 His6-Prk phosphorylates His6-Cdc25C on serine^{216}. (**a**) His6-Cdc25C protein phosphorylated *in vitro* by His6-Prk and Cdc25C protein purified from ^{32}P-labeled PC-3 cell lysates were subjected to peptide mapping as described in the Experimental procedures. The loading origins were marked as O. (**b**) Equal amounts of purified GST-Cdc25C(200–256) and GST-Cdc25C^{S216A} (200–216) fusion proteins were used as *in vitro* substrates for His6-Prk or His6-Prk^{K52R} in the presence of [γ^{32}P]ATP. The *in vitro* phosphorylated samples were analysed by SDS–PAGE followed by autoradiography

ylates Cdc25 protein (Kumagai and Dunphy, 1996). The Plx1-phosphorylated Cdc25 has an enhanced activity towards its substrate $p34^{cdc2}$ (Kumagai, and Dunphy, 1996), suggesting that Plx1 is a regulator for Cdc25. On the other hand, it has been demonstrated that Plk1 may be a substrate of $p34^{cdc2}$ and be involved in regulating the activity of anaphase-promoting complex (Kotani *et al.*, 1998), and that Plx1 is essential for degradation of a mitotic regulator and for exit from M phase (Descombes and Nigg, 1998). These observations suggest that Plk1 may have an additional, if not the primary, role during the progression of mitosis. Recently, it has been shown that c-Tak1, as well as the DNA damage checkpoint kinases Chk1 and Chk2, phosphorylate Cdc25C on $serine^{216}$ (Peng *et al.*, 1997, 1998; Matsuoka *et al.*, 1998). Phosphorylation of Cdc25C on $serine^{216}$ by *chk1* and *chk2* gene products apparently does not directly modulate its phosphatase activity, instead it promotes 14-3-3 protein binding (Sanchez *et al.*, 1997; Peng *et al.*, 1997). 14-3-3 protein then sequestrates Cdc25C in cytoplasm resulting in down-regulation of Cdc25C enzymatic activity *in vivo* (Lopez-Girona *et al.*, 1999).

We and others have previously shown that *prk* (Ouyang *et al.*, 1997) and *plk* (Lee and Erikson, 1997) genes are functional homologues of the budding yeast CDC5. We have also demonstrated that Prk's kinase activity is regulated during the cell cycle peaking at late S and G2 stages although M phase cells also contain a considerable amount of Prk activity (Ouyang *et al.*, 1997). In this paper, we report that purified His6-Prk, but not $His6\text{-}Prk^{K52R}$, is capable of strongly phosphorylating His6-Cdc25C *in vitro*. Using the co-immunoprecipitation approach, we have shown that His6-Prk interacts with His6-Cdc25C expressed in *sf*-9 cells as well as with native Cdc25C from PC-3 cells. In addition, His6-Prk is capable of phosphorylating His6-Cdc25C molecules *in vitro* at a site that is also phosphorylated *in vivo* in asynchronized cells. Considering that the activity of Cdc25C is regulated via reversible phosphorylation (Izumi and Maller, 1995; Kumagai and Dunphy, 1996), our current studies strongly suggest that Prk is a cellular regulator for Cdc25C.

It is intriguing to note that His6-Prk phosphorylates His6-Cdc25C *in vitro* on $serine^{216}$ (Figure 7). This is a site whose phosphorylation would lead to inactivation of Cdc25C activity due to sequestration in cytoplasm by 14-3-3 (Lopez-Girona *et al.*, 1999), which is apparently inconsistent with Prk's role in complementation of CDC5 mutants (Ouyang *et al.*, 1997). However, one likely explanation is that Prk may be involved in down-regulation of $p34^{cdc2}$ activity during late mitosis because considerable Prk activities remain during mitosis (Ouyang *et al.*, 1997). It has been established that activation of $p34^{cdc2}$ is controlled not only by its association with cyclin B but also by reversible phosphorylation. It is possible that inactivation of $p34^{cdc2}$ at late mitosis is also controlled by multiple mechanisms. Namely, in addition to degradation of cyclin B, inactivation of $p34^{cdc2}$ is reinforced by sequestration in cycoplasm of Cdc25C that has been phosphorylated on $serine^{216}$ by kinases such as Prk and perhaps Plk. This notion is, in fact, consistent with the observation that CDC5 *ts* mutants are arrested at late mitosis, a phenotype similar to accumulation of non-degradable cyclin B in mammalian cells. It is also consistent with the recent observation that Plx1 is required for exit from mitosis in *Xenopus* (Descombes and Nigg, 1998).

An alternative explanation of $serine^{216}$ phosphorylation of Cdc25C by Prk is that the kinase may be regulated by DNA damage checkpoint because CDC5 from the budding yeast participates in adaptation of DNA-damage-induced cell arrest (Toczyski *et al.*, 1997). One may propose that Prk expression and/or its activity may be down-regulated in response to DNA damage considering that Prk complements CDC5 *ts* mutant (Ouyang *et al.*, 1997) and thus promoting cell cycle progression. Whether or not Prk is down-regulated during DNA damage is under current investigation. On the other hand, signals other than DNA damage may also elicit $serine^{216}$ phosphorylation of Cdc25C. For example, c-Tak-1 kinase is a very active $serine^{216}$ kinase of Cdc25C (Peng *et al.*, 1998). It is yet to be demonstrated whether c-Tak-1 is activated by DNA damage checkpoint. In addition, Cdc25C is phosphorylated during G1, S, and G2 (Ogg *et al.*, 1994) in the absence of DNA damage or un-replicated DNA. Therefore, it is clear that protein kinases other than Chk1 and Chk2 are phosphorylating Cdc25C on $serine^{216}$ during normal progession of the cell cycle.

Materials and methods

Cell culture

Insect *sf*-9 cells were cultured at 27°C in Grace's insect medium supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and antibiotics (100 U/ml penicillin, 50 μg/ml streptomycin sulfate, and 50 μg/ml gentamicin, GIBCO/BRL). Dami (megakaryoblastic, ATCC, Rockville, MD, USA) and PC-3 (prostatic carcinoma, ATCC) cells were grown in RPMI1640 medium, supplemented with 10% FBS, at 37°C with 5% CO_2.

GST-Fusion protein expression and Western blotting

GST-Cdc25A, GST-Cdc25B, GST-$p34^{cdc2}$ and Prk (amino acids 334-607, Ouyang *et al.*, 1997) were expressed as glutathione *s*-transferase (GST) fusions. Fusion proteins were induced by isopropyl-β-D-thiogalactopyranoside (IPTG) and purified through affinity column chromatography using glutathione sepharose resins according to the protocol provided by the supplier. GST-Cdc25C (200-256) and its mutant GST-Cdc25 C^{S216A} (200-256) fusion proteins were kindly provided by Yolanda Sanchez. Mouse monoclonal anti-Prk antibody was produced against an epitope in the C-terminal half of Prk (Ouyang *et al.*, 1997). Mouse monoclonal anti-Cdc25C antibody was produced using the full length of Cdc25C as an immunogen. Both antibodies are available from PharMingen Inc. (San Diego, CA, USA). A rabbit polyclonal anti-Cdc25C antibody was purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). Western blot analyses were described previously (Ouyang *et al.*, 1997).

Expression and purification of recombinant human Prk

Full-length recombinant Prk was expressed using the baculoviral expression system (PharMingen) following the manufacturer's protocol. Briefly, a cDNA fragment containing the entire open reading frame of human *prk* was cloned into PVL-1393 transfer vector. To facilitate purification of recombinant Prk protein, a short nucleotide sequence coding for six histidine residues was inserted in-frame immediately

after the ATG codon of *prk* cDNA. The baculoviral expression vector BaculoGold™ DNA and the transfer plasmid PVL-1393-Prk were then co-transfected into insect *sf*-9 cells. A cDNA encoding a kinase-defective Prk (Prk^{K52R}) was generated using a site-directed mutagenesis kit (GIBCO BRL). $His6\text{-}Prk^{K52R}$ was expressed in and purified from *sf*-9 cells in the same manner as His6-Prk. Recombinant baculoviruses expressing His6-Cdc25C and GST-Wee1 were kindly provided by Dr Piwnica-Worms (Howard Hughes Medical Institute, Washington University School of Medicine, USA). Insect *sf*-9 cells expressing recombinant His6-Prk and His6-Cdc25C were harvested and lysed for analysis or for purification of recombinant proteins using Ni-NTA resins (Qiagen, Chatworth, CA, USA).

Immunoprecipitation and protein kinase assays

Immunoprecipitation and immunocomplex kinase assays were essentially as described (Ouyang *et al*., 1997). Purified recombinant His6-Prk or $His6\text{-}Prk^{K52R}$, expressed through the baculoviral expression system, was assayed for its *in vitro* kinase activity using substrates such as casein (15 μg/reaction), GST-Cdc25A (5 μg/reaction), GST-Cdc25B (5 μg/reaction) or His6-Cdc25c (5 μg/reaction, unless specified otherwise), GST-Cdc25C (200–256) (1 μg/reaction) or GST-$Cdc25C^{S216A}$ (200–256) (1 μg/reaction). The kinase reaction conditions were the same as described previously (Ouyang *et al*., 1997).

Metabolic labeling

The PC-3 cells were cultured in RPMI-1640 medium containing 10% FBS to about 80% confluence. After two washings with pre-warmed phosphate-free RPMI-1640 medium, asynchronized PC-3 cells ($\sim 5 \times 10^5$) were pulsed in the fresh phosphate-free RPMI-1640 medium containing 1.5% dialyzed FBS and 2 mCi [^{32}P]phosphate at 37°C for 4 h. After the pulse, cells were washed and lysed in the lysis buffer (Ouyang *et al*., 1997) containing 3 μM okadaic acid.

Phosphopeptide mapping

Radio-labeled Cdc25C protein was obtained either by phosphorylation *in vitro* by His6-Prk in the presence of ^{32}P or immunoprecipitated from metabolically-labeled PC-3 cell lysates. After supplementation with the carrier protein (200 μg bovine serum albumin) ^{32}P-Cdc25C protein was precipitated by the addition of trichloroacetic acid [15% (v/v) final concentration]. The protein precipitates were washed twice with cold acetone and redissolved in 50 μl NH_4HCO_3 (200 mM) containing 40 μg tosylphenylanyl chloromethyl ketone (TPCK)-treated trypsin. The dissolved protein samples were digested to completion by TPCK-treated trypsin and then dried under vacuum. The digested peptide samples were redissolved in 5 μl running buffer [5.6% (v/v) glacial acetic acid, 2.5% (v/v) formic acid, pH 1.9] and spotted onto cellulose thin layer chromatography (TLC) plates. Peptide fragments were separated first by electrophoresis using a Multiphor II unit (Pharmacia, Piscataway, NJ, USA) and then by chromatography as described (Boyle *et al*., 1991). The TLC plates were autoradiographed.

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